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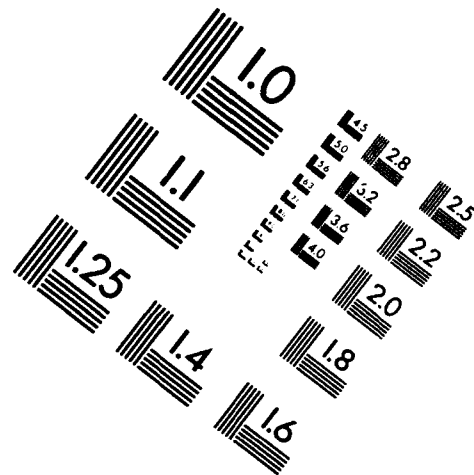
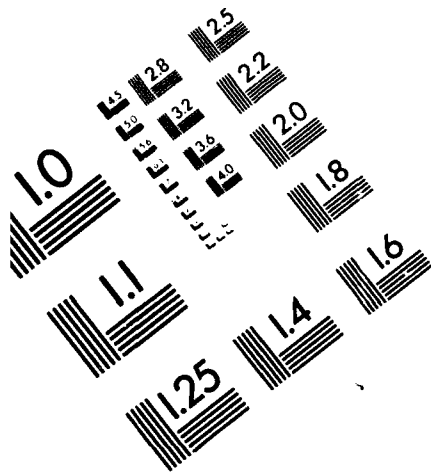


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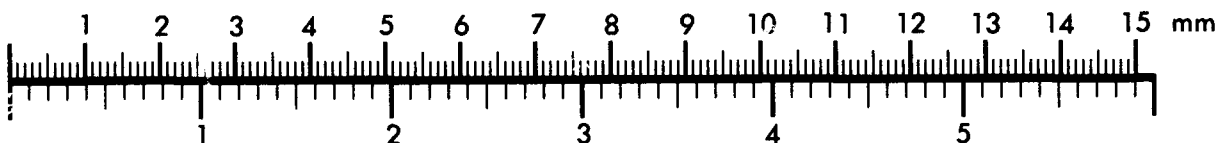
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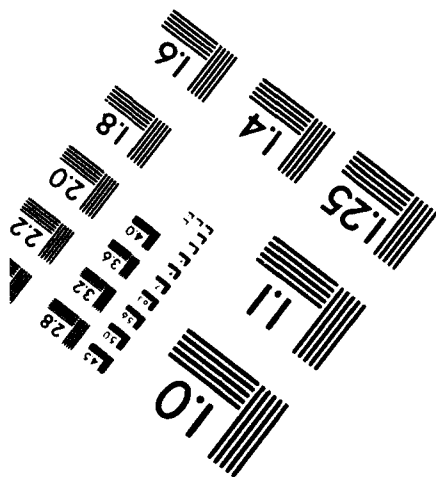
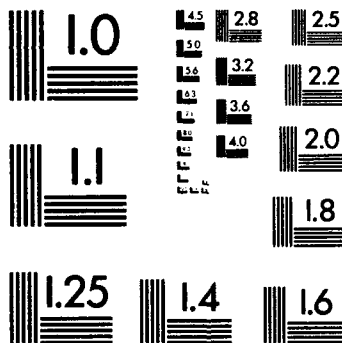
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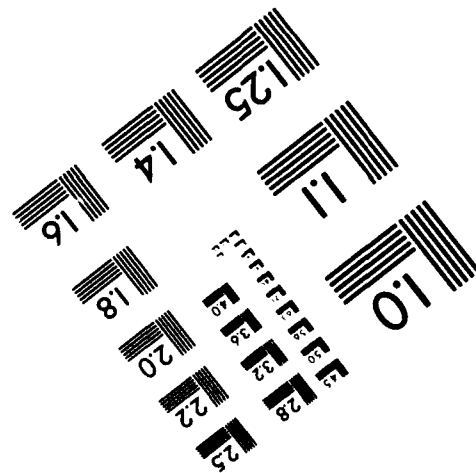
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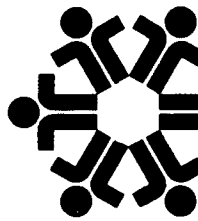


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September, 1992

Scientific Officer
Patrick Curran, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, MD 20814-50440

Ref: N00014-91-C-0044

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Dear Captain Curran:

Enclosed is the Fifth Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from May - August, 1992. If you have any questions about the Report or the research, please contact me at 404-952-1660.

Sincerely yours,

John F. Carpenter, Ph.D.
Senior Scientist

cc: Mrs. Mellars, DCMD5-GAACA
DCMAO Atlanta

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Metabolic perturbation studies on red blood cells. The central focus for this work has been testing the effects of low temperature and acidosis on energy metabolism. Our work with red blood cells supports the original alpha-stat hypothesis. That is, the degree of protonation, not pH per se, determines the relative in vivo activity of phosphofructokinase, the key regulatory enzyme of glycolysis. A manuscript describing this work is currently being reviewed for publication in the American Journal of Physiology. A copy of the submitted paper is enclosed.

We have now begun to investigate the structure-function relationships that are thought to be involved with regulating activity of phosphofructokinase in vivo. Specifically, we set out to study how alterations in quaternary structure account for enzyme inhibition under cold, acidotic conditions. The initial experiments led to some surprising, novel observations, which could greatly alter how we view the role of protein assembly state in metabolic regulation. Below we provide some background information and a brief outline of these intriguing new results.

Prior *in vitro* studies have shown that phosphofructokinase from erythrocytes (and several other mammalian cells and tissues) dissociates from active tetramers into inactive dimers and monomers at low pH. This effect follows alphastat predictions, i.e. the pH profile of polymerization state shifts toward higher pH at low temperature. This process is fully reversible upon restoration of normal alkaline pH and higher temperatures. Because the process is reversible and occurs rapidly under "physiological" conditions in vitro, changes in enzyme assembly have been proposed as an important mechanism by which PFK activity is regulated in cells. We have previously demonstrated that this is the case in ischemic myocardium, and this mechanism is generally thought to be applicable to most cells types for which their PFK was shown to dissociate in vitro.

However, our initial experiments showed that this is not the case for human red blood cells. We incubated cells under conditions that we have shown previously to inhibit the enzyme's activity greatly in vivo and which, according to the prevailing dogma, should cause the enzyme to dissociate into inactive dimers/monomers. Surprisingly, we found that there is apparently no disassembly of the enzyme. This finding has led to the hypothesis that the presence of the high concentration of hemoglobin in erythrocytes inhibits dissociation of phosphofructokinase. There is a substantial body of biophysical evidence that large, bulky polymers (hemoglobin in this case) serve to stabilize the assembled forms of proteins because the polymers are preferentially excluded from the surface of the protein. Preferential exclusion of solute leads to an increase in the chemical potential of the protein, an effect that is thermodynamically unfavorable. This effect is greater for dissociated proteins because the constituent dimers/monomers have a greater surface area than the fully assembled protein. This thermodynamic mechanism has been rigorously substantiated by Timasheff and colleagues and serves to explain the effect of solutes on protein stability, solubility and assembly state. However, prior to our observations, there has not been a single example of how preferential solute exclusion can alter enzyme structure-function relationships in vivo. Furthermore, these findings provide the first documentation, to our knowledge, that the solute environment of the intracellular milieu must be taken into account when explaining the role of protein assembly state in metabolic regulation.

To substantiate the role of hemoglobin, we have begun to purify PFK from human red cells. The partially purified enzyme does undergo pH-induced dissociation in vitro. The test of our hypothesis will be to inhibit dissociation by adding hemoglobin to the solution of the purified enzyme. To substantiate that this is a general phenomenon of bulky solutes -- which are preferentially excluded from PFK's surface due to steric hindrance -- we will also test the effects of polyethylene glycol and bovine serum albumin on pH-induced dissociation. Finally, we will test a related hypothesis that the enzyme can be inhibited by low pH, even when in the fully assembled state. That is, we will determine the pH profile for PFK activity in the presence of the bulky solutes. In this case, inhibition is due solely to the acute protonation of key histidyl residues in the stable tetramer.

Role of metabolic perturbation in damage to vascular endothelial cells. We will now apply the techniques that we have developed with red cells to study this sensitive cell type. Endothelial cells are known to be damaged by anoxia and low temperature, however, the mechanistic basis for this damage has not been determined. The relevance of these studies to our research on peripheral cold injury is two-fold. First, endothelial cells serve as a good model to determine the general mechanisms for cell death during cold ischemia. Secondly, this cell type plays a crucial role in maintaining normal hemostatic processes.

The extent of damage to these cells can range from a slight disruption of biosynthetic processes to cell death. Even in the absence of lethal damage, stress can lead to the detachment of the endothelial cells and exposure of thrombogenic surfaces in blood vessels. This latter effect appears to be especially destructive during cold-ischemic injury, because it can lead to the blockage of blood flow to the affected area. Thus, even after rewarming, ischemia and the resultant destructive conditions are maintained and lead to necrosis in the affected area.

We will start with a series of experiments to determine the acute and chronic effects of hypothermia, hypoxia, and acidosis on endothelial cells. In addition to measuring metabolic rate and energy status of the cells, we will determine intracellular calcium levels using fluorescent dyes. These experiments will determine the relative contributions to cell damage of disruption of calcium homeostasis and of perturbation of energy metabolism.

Chilling-induced platelet hypersensitivity. As we have noted in previous reports, one of the main causes of tissue damage after cold exposure appears to be the failure of vascular flow to be re-established when the tissue is rewarmed. Part of the vascular failure can be attributed to the activation and aggregation of platelets during or after cold exposure. Earlier researchers have shown that platelets will aggregate spontaneously in vitro under two different conditions: 1) after exposure to very low temperature (e.g., 0-4°C) and rewarming to 37°C; and 2) acutely, while exposed to temperatures in the range of 16-25°C. The former observation has been made with platelets in physiological buffers, whereas the latter had only been noted with platelets in ACD anticoagulant (i.e., non-physiological calcium concentrations). We have extended these studies and found that acute cold-induced hypersensitivity also occurs with platelets under physiological conditions. In addition to these direct cold-induced effects, we have documented (under physiological conditions) that at reduced temperatures

platelets are hypersensitive to agonists (e.g. ADP, thrombin, collagen). Hence, even if low temperature by itself did not lead to platelet aggregation in the affected area during peripheral cold injury, the exposure of collagen under injured endothelium and/or release of ADP from damaged cells could exacerbate platelet activation and thrombosis.

Our original hypothesis for the mechanism of cold-induced hyperaggregability was that intracellular calcium levels would be increased during hypothermia. Calcium is a key second messenger in the signal transduction pathways leading to platelet aggregation. The basis for this hypothesis was that we had shown that low temperatures induced a thermotropic phase transition of phospholipids in platelet membranes. This could directly lead to leakage of calcium down its steep concentration gradient from outside (normally about 1-2 mM) the cell to the cytoplasm (normally containing about 70-100 nM calcium). In addition, differences in the temperature coefficient for calcium pumps versus calcium channels could lead to a net increase in intracellular calcium concentrations.

We have measured intracellular calcium concentrations in platelets exposed to 4°C and then return to 37° and in platelets held at 20°C; the two treatments that cause hyperaggregability. Our results indicate that there is not a sufficient increase in intracellular calcium to cause spontaneous aggregation. For example, a typical experiment revealed that control platelets had 70.9 ± 5.1 nM Ca^{2+} at 37°C ($n = 3$) and platelets held at 20°C had 65.8 ± 6.7 nM Ca^{2+} . Platelets exposed to 4°C and then rewarmed to 37°C had 94.0 ± 3.6 nM Ca^{2+} . The typical range for Ca^{2+} levels in resting platelets is 70 to 100 nM. (During activation calcium levels increase to several-hundred nM.) Spontaneous aggregation tests on the same platelet preparations indicated that after 30 minutes the 37°C controls aggregated only 7%, while platelets incubated at 20° aggregated to 62% of maximum. Those exposed to 4°C and then re-warmed to 37°C aggregated 100%. Thus, it appears that the effect of temperature on platelet aggregation is not mediated through a rise of calcium during exposure to low temperatures.

However, we still need to test whether Ca^{2+} levels rise higher upon addition of agonists after 4°C exposure or during 20°C incubation. This observation would help explain why platelets are hypersensitive to agonists during or after cold exposure. We are currently generating agonist dose-response curves for platelets at 37 and 22°C and measuring intracellular calcium levels under identical conditions.

An extensive review of the literature, in combination with our observation of thermotropic membrane phase transitions in platelets, has led to the formulation of a new hypothesis for cold-induced platelet hyperaggregability. Two potent agonists for platelets are arachidonic acid and its metabolite thromboxane. Arachidonic acid production from phospholipids is catalyzed by phospholipase A₂. In vitro studies have shown that the maximum catalytic activity is seen when the enzyme is presented with substrate phospholipids that are in mixed phase (i.e., the mixture of gel and liquid crystalline states noted at the phase transition temperature). We hypothesize that at low temperature the in vivo thermotropic phase transition of platelet membranes leads to activation of phospholipase A₂ and concomitant production of the arachidonic acid and thromboxane. Upon stirring, these agonists lead to platelet aggregation. We will test this hypothesis by measuring thromboxane production rates in platelets incubated at

20, and in platelets exposed to 4°C and then rewarmed to 37°C.

Once the mechanism by which spontaneous aggregation occurs is understood, it may be possible to modify platelets to make them less susceptible to chilling-induced aggregation. For example, cellular membranes can be fluidized by the addition of agents such as α -tocopherol (vitamin E). Thus, addition of tocopherol to platelets may allow them to be cooled to lower temperatures before the membranes enter a mixed phase. In a recent publication, it was observed that (under isothermal conditions) tocopherol analogues are inhibitors of phospholipase A2 in vitro. We will test to see whether this effect is operative in vivo under hypothermic conditions and whether it is mediated by changes in membrane fluidity.

Potential applications of antifreeze protein to cold injury. We have completed our studies on using antifreeze protein to attenuate ice recrystallization damage to frozen cells. A paper describing this work has been accepted for publication in the Proceedings of the National Academy of Science. A copy of the manuscript is enclosed.

We have also initiated a collaborative study with Dr. Lewis Teperman (Chief of Transplant Surgery) at the New York University Medical Center. Dr. Teperman and his colleagues will be testing the effects of antifreeze proteins on cell survival during hypothermic storage at temperatures near or slightly below 0°C.

Effect of acidosis and ionic environment on skeletal muscle fibers. Our collaborators (Profs. Nosek and Godt) at the Medical College of Georgia have continued to assess the influence of perturbing conditions on skeletal muscle fiber function. Fatigue or ischemia of skeletal muscle is accompanied by an increase in intracellular inorganic phosphate (Pi) and a decrease in pH. We have previously reported on how these two changes in the milieu decrease the contractile function of skinned fibers at room temperature (22°C). We found that at 22°C fast-twitch skeletal muscle is sensitive to the acidic form of Pi. Thus, the depressant effect of increased Pi and reduced pH on muscle force in response to stimulation will be greater than expected from the simple additive effects of Pi and pH. We have now investigated the temperature dependence (37°C - 7°C) of the Pi and pH effects on skinned fast-twitch rabbit psoas muscle fibers. We found that: 1) The magnitude of the depressant effects of both 30 mM Pi and a change in pH from 7.0 to 6.2 increased as the temperature of the medium was lowered. That is, under hypothermic conditions these ionic changes would be much more perturbing of muscle function than they would be at 37°. 2) The synergistic effect of Pi and pH was present at both 37°C and 22°C, but absent at 7°C.

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